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13. ABSTRACT *(Maximum 200 Words)*

We are developing gene therapy vectors derived from purified polyomavirus capsid proteins. We hope to achieve selective targeting of the vectors by expressing on their surface, sequences that bind receptors present on the surface of cancer cells. We have modified the polyomavirus VP1 capsid protein to contain sequences derived from urokinase plasminogen activator (uPA) responsible for binding to the urokinase plasminogen activator receptor (uPAr) and with sequences capable of binding to the ErbB2 receptor. These modified VP1 proteins have been expressed in insect cells. Methods for their purification have been developed, and their capacity to assemble into virion-like particles (VLPs) assessed by electron microscopy and by sedimentation analyses. We have demonstrated self-assembly of VP1 proteins containing both uPA and ErbB2 ligands into VLPs, which should enhance the specificity of binding to cells expressing both receptors. We have determined that the modified VP1 loses its capacity to bind to sialic acid, a major component of the normal binding of polyomavirus to cells, and we are presently attempting to determine the specificity of the homotypic and heterotypic VLP for cancer cells.

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Introduction: The most life-threatening aspect of cancer is its capacity to invade normal tissue and to establish new foci of tumor cells at distant sites. While there has been great progress in understanding the genetic and cellular mechanisms involved in the conversion of normal cells to metastatic tumor cells, less progress has been made in utilizing what has been learned to reduce cancer morbidity and mortality. The objective of this work is to develop novel gene therapy vectors selective for metastatic cells. Selectivity will rely upon metastatic cells expressing urokinase plasminogen activator and erbB2 receptors to which the vectors can bind.

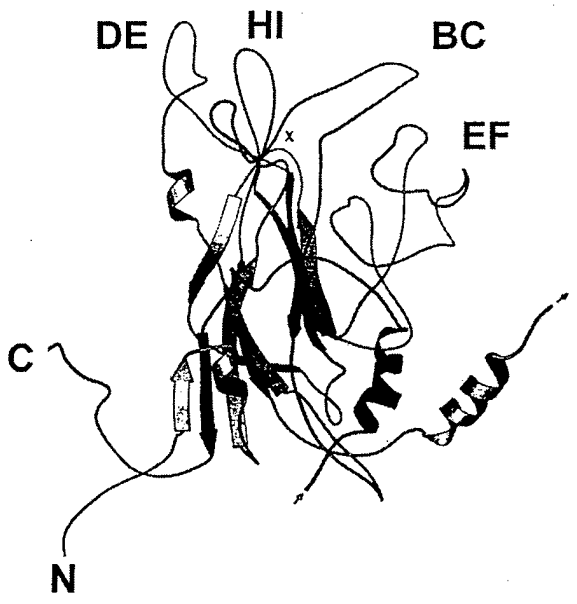
Research Accomplished:

1. Construction of modified VP1 gene(s)

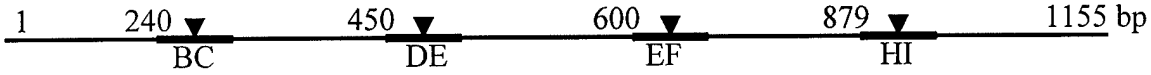
The polyomavirus major capsid protein VP1 will self-assemble to make virus-like particles (VLP) without involvement of other capsid proteins (Salunke DM. *et al.* 1986; An K *et al.*, 1999). The relative simplicity of self-assembly makes polyomavirus an attractive candidate for a gene therapy vector.

VP1 is composed of 384 amino acids and its 3-dimensional structure has been reported (Soeda E *et al.* 1980). Four loops, BC, DE, EF and HI are exposed on the outside surface in capsomere structure and hence may not be involved in forming virus-like particles. We selected those four loops as target sites to insert foreign peptides.

Structure of polyoma VP1 protein and sites of foreign peptide insertion



It has been shown that amino acid sequence of 13-32 of uPA is responsible for its binding to uPAR (Appella *et al.* 1987). Consequently, we have chosen to insert residues 10-34 of uPA into the VP1 protein so that it might interact with the uPAR receptor. Goodson RJ *et al.* (1994) selected 15-mer peptides with high affinity for uPAR using random bacteriophage display. The selected peptides have two short conserved sequences, neither of which is found in uPA protein. One such peptide, present in clone 20, (AEPMPHSLNFSQYLWYT), showed a higher affinity for the uPA receptor than original uPA peptide. We have inserted this peptide into the loops of VP1. Karasseva et al (unpublished) have identified a hexapeptide (KCCYSL) that binds to ErbB-2 with high specificity, and we have inserted this into VP1. To help evaluate the effect of insert size on VP1 structure, a FLAG epitope was inserted in the HI loop of VP1. Self-assembly of VP1: FLAG protein has been compared with that of other modified VP1 proteins.

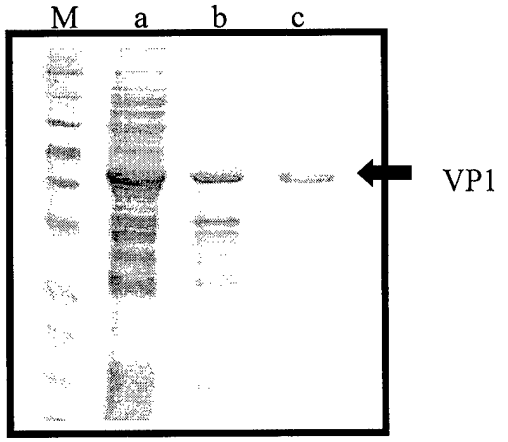


VP1 protein: (▼ : Site of foreign peptide insertion)

2. Cloning of wild type and modified VP1 gene(s) into baculovirus and protein expression:

Wild type and modified VP1 gene(s) were cloned into the pFastBac expression plasmid and transformed into DH10Bac competent cells containing Bacmid plasmid DNA. Recombinant bacmids containing wild type or modified VP1 gene(s) were purified and transfected into Hi-5 insect cells to obtain recombinant baculovirus.These were amplified to obtain high titer virus stocks. To produce VLP, confluent monolayer of Hi-5 cells were infected with recombinant baculoviruses and incubated for 4 days. Protein production was evaluated by western blot analysis.

1. Infection of Hi-5 cells with baculovirus (1 x 10e7 cells/T150 flask, m.o.i = 10)
2. Incubation for 3.5 days(2 % FCS)
3. harvest and sonication sedimentatio (40sec x 3, take supernatant: **a)**
4. Sucrose sedimentation (sucrose 20%, SW 28 rotor, 27K, 3 hr: **b**)
5. Sucrose sedimentation velocity (Sucrose 10-50%, SW40 rotor, 35k, 2hrs: **c**)

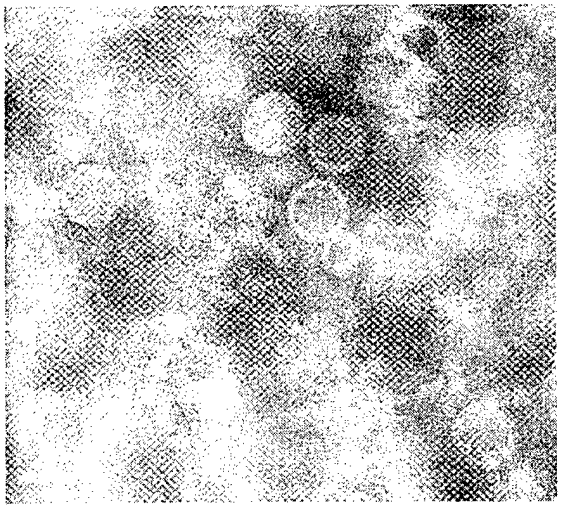


SDS Page of VP1 Purification

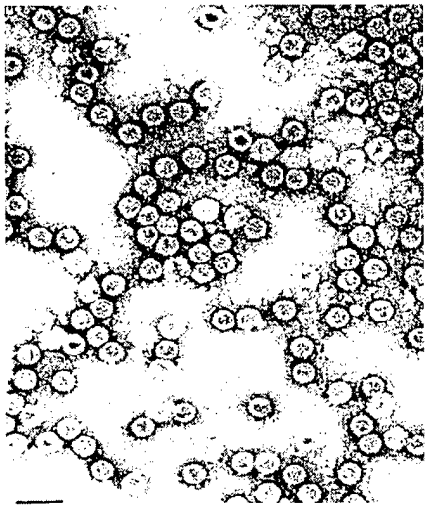
3. Purification of VLP and transmission electron microscopy

After harvesting the infected cells by low speed centrifugation, Hi-5 cells were disrupted by brief sonication and the lysate was centrifuged at 10.000g for 30minutes, with the supernatant being saved. The pellet was re-extracted by sonication and centrifuged same as above. The supernatant was saved and combined with the original supernatant. VLPs were concentrated through 2ml of a 20% sucrose shelf and centrifuged at 35,000rpm for 120 minutes in a Beckman SW40 rotor. The pellet containing the partially purified VLPs was resuspended in small volume of buffer and layered on top of a preset cesium chloride gradient (1.35g/ml-1.23g/ml) and centrifuged at 33,000rpm for 15 hours in a SW40 rotor and then fractions were collected. The fraction containing VLP was dialyzed and used for transmission electron microscopy.

Electron microscopy images of VLPs



VLPs made from insect cell expressed VP1



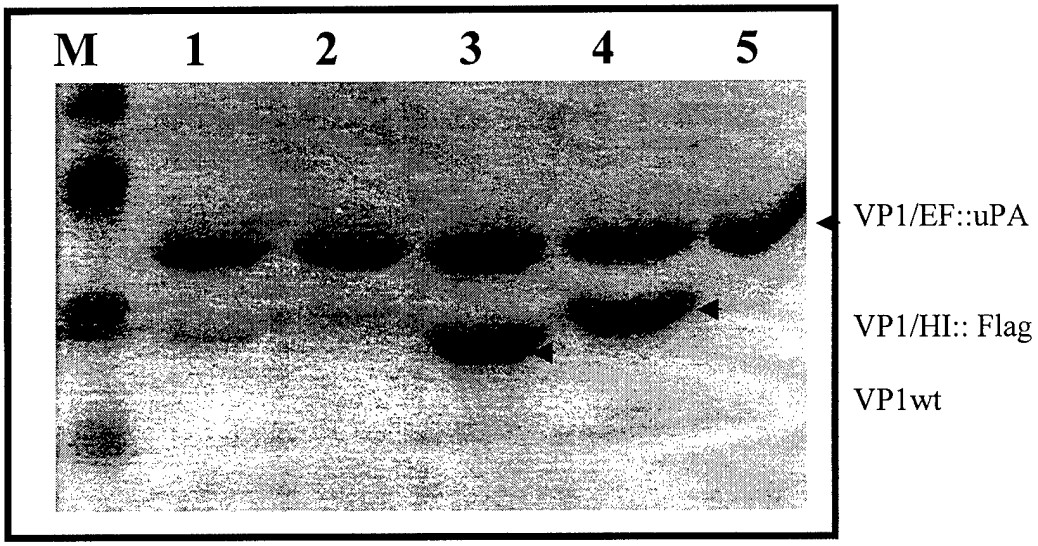
Native polyoma virus

4. The solubility of modified VP1 proteins was examined by measuring the distribution between supernatant and pellet. Hi-5 cells were infected with baculoviruses which express each modified VP1. Cells were harvested at 84 hrs pi and sonicated, followed by high speed centrifugation (15,000g for 30 min). SDS PAGE and western blots were performed to evaluate the distribution of modified proteins. All of the uPA and ERB-B2 inserts into the EF loop of VP1 yielded soluble proteins, whereas these inserts into the BC, DE and HI loops generally produced insoluble proteins. Insertion of the Flag peptide into the EF loop produced soluble proteins.

5. The reactivity of the modified proteins against anti-uPA antibodies was determined. American Diagnostica Antibody #3921 reacted strongly against uPA sequences introduced into the EF loop, indicating the native structure of uPA was retained.

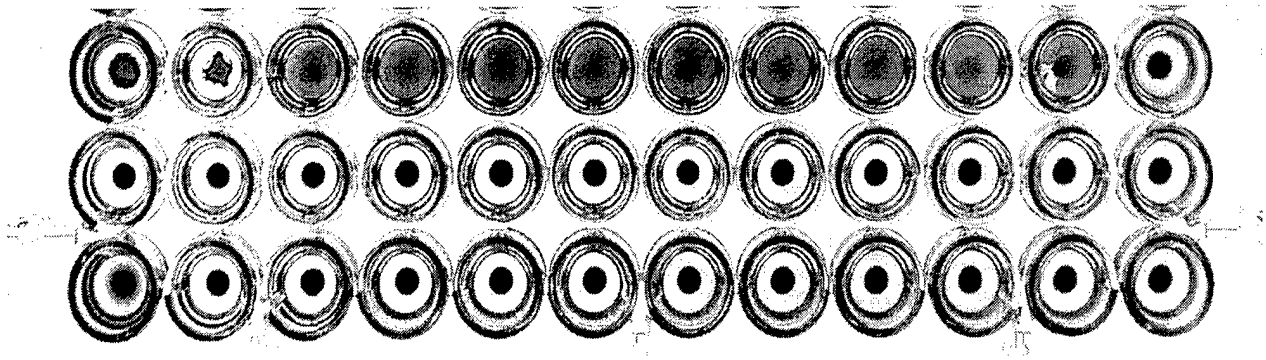
6. To produce heterotypic VLPs, Hi-5 cells were co-infected with baculoviruses expressing VP1/wt and VP1/EF::uPA1-60. VP1 proteins were purified by pelleting through sucrose and were observed to co-sediment.

To obtain firm evidence that the VLPs contained both species of VP1, heterotypic VLPs containing Flag ligand and uPA 1-60 were prepared by co-infection of Hi-5 cells and immunoaffinity purified with Anti-Flag antibody. These VLPs were demonstrated to contain the uPA 1-60 insert by subsequent immunoprecipitation with Anti-uPA antibody #3921.



Cells Infected with:
1. VP1/wt
2. VP1/HI:: Flag
3. VP1/wt + VP1/EF::uPA 1-60
4. VP1/HI:: Flag + VP1/EF::uPA 1-60
5. control

7. To determine whether the insertion of the ligands into VP1 affected adsorption of the virus to sialic acid receptors, hemagglutination assays were performed with the VLPs. The assay indicated that the modified VLPs (rows 2&3) did not hemagglutinate.



8. Binding of the modified VLPs to DU145 cells expressing uPAR indicates that VLPs containing the uPA ligand bind more avidly than VLPs containing the Flag ligand, whereas wild typr VLPs bind even more strongly (because of their affinity for sialic acid). These experiments are being refined to determine that the basis for the preferential binding of the VLPs with the uPA ligand can be competed by uPA or ATF.

9. Attempts to incorporate chromatin into the VLPs have begun, and will be the focus of the efforts during the next year.

Key research Accomplishments to date:

1. We have expressed and purified polyomavirus VP1 proteins containing inserts derived from sequences that bind uPAR and Erb-B2.

2. We have assembled virus like particles (VLPs) from these modified proteins.

3. We have shown the inserts are expressed on the surface of the VLPs in a form that can be detected by antibodies.

4. We have expressed VLPs containing inserts that bind to both uPAR and Erb-B2.

5. We have demonstrated that the modified VLPs lose the capacity to bind to normal cells.

6. We have demonstrated affinity of the modified VLPs for cells expressing uPAR.

Reportable Outcomes:

1.Development of clones that express chimeric VP1 proteins.

2.Development of procedures for the purification and self-assembly of modified VP1 proteins

3.Progress toward completion of a PhD degree by Mr Young-Shin, graduate student.

4. These results have been reported at an international conference, the 2001 ICRF/UCSF DNA Tumor Virus Meeting.

Conclusion: We have essentially completed Technical Objectives 1 & 2 of the Statement of Work. This year we will continue to work to achieve a modified Technical Objective #4. Achievement of Technical Objective #3 will require additional effort.

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